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Long-term impacts of de-oiled two-phase olive mill waste on soil chemical properties, enzyme activities and productivity in an olive grove

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ABSTRACT

Soils in semi-arid Mediterranean areas are generally characterized by low organic matter content and are subjected to progressive degradation and deterioration of workability. Because de-oiled two-phase olive mill waste (DW) contains an important level of organic matter content, its recycling as organic amendment or fertilizer may be an alternative for its disposal that also improves soil quality and productivity. A ten-year field study was conducted to evaluate the long-term sustainability of raw de-oiled two-phase olive mill waste (DW) disposal as a soil amendment on an olive grove in Elvas, Portugal. The soil was amended with DW at rates of 0, 27, and 54 Mg ha^{-1}, dry weight equivalent, for eight years, with cumulative and residual effects being assessed in the last year and two years after the last application. The DW amendments significantly increased the olive yield only in the residual year at the 27 Mg ha^{-1} application rate. Long-term applications of DW to soil led to positive cumulative and residual effects on the soil's chemical (total organic carbon and its humified fractions, total N, available P, and K), properties. Simultaneously, dehydrogenase, urease, β-glucosidase, alkaline phosphatase, and arylsulfatase activities increased even at the higher DW application rates. Electrical conductivity rose significantly with DW application, especially in the residual year, ranging from 0.513 dS m^{-1} for the unamended soil to 1.89 dS m^{-1} at the 54 Mg ha^{-1} application rate. The addition of raw DW to an olive grove may be considered to be a good strategy for recycling this waste, converting it into a resource that could be used for a long time as organic amendment without negatively affecting yield, while improving many soil properties. However, the greatest concern regarding the long-term use of DW is the risk of soil salinity, especially if application rates are greater than 27 Mg ha^{-1}.

1. Introduction

Olive oil extraction is one of the most traditional agricultural industries, with great economic importance, in most Mediterranean countries (Owen et al., 2000). In Spain alone, the largest olive oil producer in the world, more than 4 000 000 Mg of organic slurry is generated annually from the continuous centrifuge two-phase process (two-phase olive mill waste, OW). After drying the OW, the remaining oil still present in this waste is usually extracted with hexane, leaving a solid residue – de-oiled two-phase olive mill waste (DW). The utilization of most of the OW and DW treatments that have been proposed remains uncertain for economic and technical reasons (Hanifi and El-Hadrami, 2009). Traditionally, DW has mainly been used as fuel for small boilers. However, the enactment of international regulations limiting the emission of CO_2 has lately been restricting this practice, so that new solutions are required for appropriate disposal or recycling.

Low organic matter content is a common feature of Mediterranean soils. It causes deterioration of workability, and contributes to limiting fertility and productivity (Albaladejo et al., 1994). Moreover, the continuous decomposition of organic matter in long-term arable Mediterranean soils may lead to soil degradation with the consequent inability to ensure sustainable production (Hemmat et al., 2010). Therefore, agricultural practices based on periodic inputs of organic amendments are strongly recommended for Mediterranean agro-ecosystems. Because traditional organic soil amendments, such as farmyard manure, are locally scarce (Saviozzi et al., 1999) and DW contains a large amount of organic matter, DW might be useful as an amendment for agricultural soils, potentially lowering the need for inputs of N, P, and K fertilizer, and improving a range of soil properties (López-Piñeiro et al., 2008). This strategy could be of especial importance for the maintenance of the olive grove ecosystem in which the return of the organic matter to the soil, apart from its positive effect of storing C in the

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soil, would help to prevent their well-known problems associated with erosion (Pleguezuelo et al., 2009). Moreover, DW might be more amenable to biological upgrading than OW due to its greater stability during mid-term storage and better textural properties (Roig et al., 2006; Sampedro et al., 2009).

Several studies have focused on the use of composted OW as a soil amendment or fertilizer, although most were carried out under short-term and/or greenhouse conditions (e.g., Altieri and Esposito, 2008; Fornes et al., 2009; Madejón et al., 2001). Few studies however have investigated the beneficial effects of fresh OW on restoring soils and crops (Brunetti et al., 2005; López-Piñeiro et al., 2006). Even fewer have looked at applying uncomposted DW directly as an organic amendment, although several researchers have found that raw organic material may be the most effective amendment for improving soil physical and mechanical properties, and consequently increasing the workable range and restoring productivity to degraded soils. Thus, Kavdir and Killi (2008) reported that fresh OW had great potential to improve the soil structure of coarse textured soils. However, Lozano-García et al. (2011) found that, although OW application could improve several physical soil properties and reduce soil erosion, it could also reduce available water capacity in olive grove soils. In a short-term laboratory study, direct application of DW has been found to increase organic C content, improve nutrient levels and aggregate stability, and increase wheat yield up to 202% relative to controls (López-Piñeiro et al., 2008). Although less known however, application of DW may also lead to immobilization of the soil's mineral N and P, thereby creating nutrient deficiencies and reducing crop yield, particularly in degraded soils (López-Piñeiro et al., 2008). Although it is known that the addition of organic amendments may affect soil's microbial biomass and enzyme activities, which can greatly influence plant productivity (Fernández et al., 2009), little is known about the effects of DW on microbiological and biochemical factors. Sampedro et al. (2009) in a short-term laboratory study found that direct DW application was not as toxic to soil microorganisms as they expected, suggesting that DW pre-treatment might not be necessary.

Although the application of fresh DW can mean important advantages in terms of time and costs, its indiscriminate use over a long time could result in an accumulation of toxic compounds such as salts and phenolic substances with detectable negative effects. Though olive groves would appear to be suitable for DW application, there is very little information available on the agronomic and environmental sustainability of this practice. Moreover, to the best of our knowledge, there have no been published studies comparing the cumulative and residual effects of repeated applications of raw DW on soil properties, nutrient status, and olive yield under long-term field conditions. Such information would be useful to validate previous laboratory data and to ensure the sustainable use of this abundant resource as organic amendment or fertilizer, especially in degraded Mediterranean agro-ecosystems.

The objectives of this field study were to: (1) evaluate the cumulative effects (8 years) of repeated applications of DW on the nutritional status and production of an olive grove; (2) measure the influence of repeated applications of DW on a soil's chemical, and biochemical properties; and (3) determine the residual effects of DW application on olive yield and soil properties.

2. Materials and methods

2.1. Experimental design

A field experiment was conducted in Elvas, Portugal (38°53′N; 7°9′W; 290 m above sea level) on an olive grove (*Olea europaea* L.) with conventional tillage practices and amended or unamended with DW for eight successive years (from 1999 to 2006). The soil, classified as a Cutanic Luvisol (ISSS-ISRIC-FAO, 1994), consisted of 19.7% clay, 19.7% silt, and 60.6% sand. The climate is semi-arid Mediterranean with an average annual rainfall of 500 mm occurring mostly in autumn and spring and a mean annual temperature of 16.7 °C. The DW was obtained from the UCASUL oil industry located in Beja (Portugal), which employs chemical and heat treatment to obtain a second-extraction olive-oil. Its main properties were as follows: pH 5.30, 516 g kg^{-1} organic carbon, 24.0 g kg^{-1} total N, 1.94 g kg^{-1} total P, 12.5 g kg^{-1} total K, 14.6 g kg^{-1} water soluble phenols, 5.30 dS m^{-1} electrical conductivity, 23.7, 16.7, and 21.5 g kg^{-1} lignin, hemicellulose, and cellulose, respectively, and 5.40% moisture content.

The experimental design consisted of 9 plots in the olive grove, with amendments made in a complete randomized design with three replicates per treatment. Each plot consisted of 12 trees in 4 × 3 orientation, in which only the central two trees were used for sampling. The three amendment treatments consisted of 27 (DW27) and 54 (DW54) Mg DW ha^{-1}, dry weight equivalent, and a control (DW0) (unamended soil). Amendments were applied annually in February (from 1999 to 2006), manually spreading the waste on the soil surface, and then incorporating it to a depth of about 15 cm with mouldboard plowing.

Four soil subsamples from each olive grove plot were taken randomly at a 25-cm depth in December 2006 and December 2008. Field-moist and air-dried samples were passed through a 2-mm sieve and stored at 4 °C until analysis. Since no DW amendments were added after 2006, the measurements made in 2006 and 2008 represented the "cumulative" (DW0C, DW27C, and DW54C samples) and "residual" (DW0R, DW27R, and DW54R samples) effects, respectively.

2.2. Analyses of the soil and DW

The pH was measured in 1:1 (w/v) soil/water and 1:5 (w/v) OMW/water suspensions using a pH-meter with a combination electrode. Electrical conductivity (EC) was measured in saturated soil samples (USDA, 1954). Total organic C content (TOC) was determined by dichromate oxidation (Nelson and Sommers, 1996). Water-soluble organic C (WSOC) was extracted with de-ionized water at 3:1 (water to soil) and 100:1 (water to OMW) ratios. Humic and fulvic acids (HA and FA) were extracted using a solution of 0.1 M Na$_4$P$_2$O$_7$ + NaOH and a ratio of extractant to soil sample of 10:1. The supernatant was acidified to pH 2 with H$_2$SO$_4$ to precipitate humic acids. The WSOC and the TOC associated with each fraction of HA (CHA) and FA (CFA) were determined by dichromate oxidation at an absorbance of 590 nm (Sims and Haby, 1971). The polymerization grade (PG) was calculated as (CHA/CFA). Total N content was determined by the Kjeldahl method (Bremner and Mulvaney, 1982). Mineral N as N–NO$_3$ was extracted using 2 M KCl solution (Keeney and Nelson, 1982) and measured by steam distillation with MgO and Devarda's alloy. Available P was determined according to the method of Olsen et al. (1954) using the ammonium molybdate-ascorbic acid method described by Murphy and Riley (1962). Available K was extracted by 1 M NH$_4$OAc at pH 7 and was assayed by atomic absorption spectrophotometry. Soil texture was determined by sedimentation using the pipette method (Gee and Bauder, 1998).

The water content of the DW was calculated from weight loss after oven drying to a constant weight at 105 °C. Total P and K in the DW were extracted by Na$_2$S$_2$O$_7$ fusion (Hossner, 1996). Water soluble phenolic substances were determined by the Folin–Ciocalteu colorimetric method (Box, 1983). Cellulose, hemicellulose, and lignin were determined using the acid and neutral fibre detergent method (Goering and Van Soest, 1970).

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2.3. Enzyme activities

Dehydrogenase (DH) activity was determined by the method of Trevors (1984) modified by García et al. (1993). One gram of soil was incubated for 20 h at 20 °C in the dark with 0.2 ml of 0.4% 2-*p*-iodophenyl-3 *p*-nitrophenyl-5 tetrazolium chloride (INT) as substrate. At the end of the incubation the iodonitrotetrazolium formazan produced was extracted with 10 ml of methanol and the absorbance measured at 490 nm. The DH and other activities were determined in triplicate.

To assay urease (UR) activity, 2 ml of 0.1 M pH 7.0 phosphate buffer and 0.5 ml of 1.066 M urea were added to 0.5 g of soil and incubated for 1.5 h at 30 °C. The ammonia released in the hydrolytic reaction was measured spectrophotometrically at 636 nm (Kandeler and Gerber, 1988; Nannipieri et al., 1980).

The activity of β-glucosidase (GLU) was determined by incubating 1 g of soil with 4 ml of 25 mM 4-nitrophenyl-β-d-glucanopyranoside in 0.1 M modified universal buffer (MUB) pH 6.0 (Tabatabai, 1982). For the determination of phosphatase (PHO) activity, 4 ml of 25 mM 4-nitrophenyl phosphate MUB pH 11 was added to 1 g of soil (Tabatabai and Bremner, 1969). For the determination of arylsulfatase (ARS) activity, 4 ml of 5 mM 4-nitrophenyl sulfate in 0.5 M acetate buffer pH 5.8 was added to 1 g of soil (Tabatabai and Bremner, 1970). The soils were incubated for 1 h at 37 °C. The samples were then cooled to 2 °C for 15 min to stop the reaction, and the *p*-nitrophenol produced in the enzymatic reactions was determined at 400 nm, 398 nm, and 410 nm for GLU, PHO, and ARS, respectively. Blank assays without soil and without substrate were performed at the same time as controls.

2.4. Crop measurements

The effect of different treatments on the nutritional status of the olive trees was determined by leaf analysis of a composite sample of 100 leaves per plot (Beutel et al., 1983) collected in July 2006 and 2008. In the laboratory, leaves were washed with 0.03% triton X-100, rinsed with de-ionized water and oven dried for 72 h at 65 °C. Dried leaf samples were ground and wet-ashed in a block digester using an H_2SO_4–H_2O_2 mixture (Lowther, 1980) to determine the P, N, and K concentrations. Nitrogen content was determined by the Kjeldahl method; phosphorus was determined colorimetrically using the method described by Murphy and Riley (1962); and potassium by atomic absorption spectrophotometry. Tree crops were harvested manually (Ravetti, 2008), and olive production was determined by weighing the central trees' yield in each replicate plot.

2.5. Statistical analyses

Statistical analyses were carried out using SPSS 11.5 for Windows (SPSS Inc., 2002). The experimental design was a completely randomized design with three replicates. A repeated measure ANOVA was conducted for selected soil and crop parameters to assess the effects of treatment. The data were analyzed differently by separating out effects following eight years of DW application from those observed two years after the last application. All pairwise multiple comparisons were performed using the Tukey test. Differences between treatment means were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Olive tree yields and leaf nutrient concentrations

In 2006, following eight years of annual DW application, non-significant differences were obtained in olive production (Table 1) despite high concentrations of potentially toxic organic compounds (e.g., phenolic substances) in amended soils (Table 2).

Table 1
Cumulative and residual effects of repeated applications of de-oiled two-phase olive mill waste (DW) on yield and leaf N, P, and K concentrations.

	Units	Year 2006			Year 2008			Analysis of variance		
		DW0C	DW27C	DW54C	DW0R	DW27R	DW54R	T[a]	S[a]	T × S
Yield	($Mg\,ha^{-1}$)	8.55a	8.59a	8.75a	7.13a	8.10b	7.64a	*	*	*
N concentration	($g\,kg^{-1}$)	12.3a	14.0b	15.1c	12.0a	15.6b	17.3c	**	*	NS
P concentration	($g\,kg^{-1}$)	1.24a	1.82b	1.78b	1.75a	1.92b	2.13c	**	**	*
K concentration	($g\,kg^{-1}$)	7.83a	8.49b	8.40b	7.58a	10.6b	10.2b	*	*	*

[a] T: treatment; S: sampling time; values with the same letter within a row, for a given sampling date, are not significantly different at a $P < 0.05$ level of probability;
[*] Significant at 0.05 probability levels.
[**] Significant at 0.01 probability levels. NS, is not significant.

Table 2
Cumulative and residual effects of repeated application of de-oiled two-phase olive mill waste (DW) on selected soil properties (0–25 cm depth).

	Units	Year 2006			Year 2008			Analysis of variance		
		DW0C	DW27C	DW54C	DW0R	DW27R	DW54R	T[a]	S[a]	T × S
Organic carbon	($g\,kg^{-1}$)	11.07a	35.70b	55.90c	10.26a	33.50b	48.10c	***	NS	NS
Total N	($g\,kg^{-1}$)	1.53a	3.20b	4.71c	1.12a	2.84b	4.16c	***	*	*
NO_3–N	($mg\,kg^{-1}$)	6.79a	18.3b	22.1c	5.91a	15.4b	19.7b	***	*	*
WSOC	($mg\,kg^{-1}$)	142a	466c	615c	122a	236b	296b	***	**	*
Humic acid	($g\,kg^{-1}$)	1.348a	1.702b	2.569c	1.268a	3.002c	4.320d	***	**	**
Fulvic acid	($g\,kg^{-1}$)	0.873a	1.439b	2.032c	0.830a	0.916a	1.201b	***	**	*
PG[a]		1.54c	1.18b	1.26b	1.52c	3.27d	3.59d	**	***	*
pH		8.00c	7.94bc	7.52b	8.03c	7.77b	7.55b	*	NS	NS
Available P	($mg\,kg^{-1}$)	14.0a	47.9b	60.7b	10.7a	51.8b	78.3c	***	*	*
Available K	($mg\,kg^{-1}$)	351a	1053b	1989b	376a	1404b	1878c	***	NS	NS
WSP		16.2a	44.9b	103c	12.4a	18.0a	34.5b	**	**	*

[a] T: treatment; S: sampling time; values with the same letter within a row, for a given sampling date, are not significantly different at a $P < 0.05$ level of probability.
[*] Significant at 0.05 probability levels.
[**] Significant at 0.01 probability levels.
[***] Significant at 0.001 probability levels. NS, not significant.

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Similar results have been reported by Altieri and Esposito (2008) in another field study but using OW as organic amendment, and conducted over a shorter time frame (5 years) with lower amendment application rate (more than 3 times less than in our experiment). However, after eight years of continued application of OW to the same soil, López-Piñeiro et al. ~~(in press)~~ found that olive yields increased by about 17% in those OW-amended soils. In the residual year 2008, DW application increased yields, although this increase (13% compared to the control) was statistically significant only for the lower DW rate (DW27R) (Table 1). The results are similar to those reported previously regarding residual effects of DW application in two Mediterranean soils, although those were obtained in a greenhouse experiment and using a wheat crop (López-Piñeiro et al., 2008). Also similar, although using residues from a three-phase decanter centrifugation process for the oil extraction, were the results reported by Brunetti et al. (2005, 2007) who concluded that the increased total content of acidic functional groups in the amended soil's humic acids affected the wheat grain yield positively in a short-time field experiment. However, López-Piñeiro et al. ~~(in press)~~ reported that the increase in olive yield provided by OW in the residual year was significantly greater (>23%) than the increase observed in the present study (13%, Table 1) for DW-amended soils. This indicates the importance of the specific characteristics of the organic amendment or fertilizer in determining crop productivity.

Despite the studied soil's high natural fertility (in the chemical sense, Table 2), the nutritional status of the olive trees in the unamended soil was characterized by N and K values below the threshold for the sufficiency range in both the cumulative and the residual years (Table 1) (Marín and Fernández-Escobar, 1997). This result was to be expected since these soils were left unfertilized for the ten years of our study. In contrast, the leaf N (except for DW27C treatment), P, and K concentrations in the amended soils were above the sufficiency threshold in both the cumulative and the residual years, indicating that the DW amendment may compensate the lack of mineral nutrition in the soil. After eight years of repeated DW application, leaf N concentrations increased significantly ($P < 0.05$) with increasing DW rate (Table 1), indicating that the expected N immobilization did not occur in our study. This positive effect was more evident in the residual year.

Similarly to N, a significant ($P < 0.05$) positive effect on leaf P and K concentration was also observed after repeated raw DW applications. At the higher loading rate of DW, the relative increases in leaf P were 43% and 22% for the cumulative and residual years, respectively, compared with the control. This suggests that not all the P was immobilized during the experiment, despite the high C/P ratio shown by this waste. Leaf K content also increased after repeated application of DW (Table 1), with this increase being more evident in the residual year.

3.2. Chemical properties of the soil

In the control soil, the organic carbon content was low as is typical for Mediterranean agricultural soils (Table 2). As a consequence of long-term DW application, the TOC significantly ($P < 0.05$) increased from 11.0 g kg^{-1} in the control to 35.7 and 55.9 g kg^{-1} for DW27C and DW54C treatments, respectively. Two years after the last DW application, TOC remained relatively constant in DW-amended plots (especially in DW27R treatment), suggesting moderate to high amounts of DW were capable of building stable organic matter pools resistant to decomposition.

Similarly to TOC, repeated raw DW applications led to significant positive effects on HA, FA, and WSOC (Table 2). Compared to the control, amended soils had greater HA, FA, and WSOC, with higher percentages attained at the 54 Mg ha^{-1} application rate (91%, 132%, and 333% greater for HA, FA, and WSOC, respectively). Two years after the last DW application the HA content significantly increased, while FA and WSOC contents decreased (Table 2). Thus, compared with the control, the HA increased by factors of 1.9 and 3.4 for the cumulative and residual years, respectively, at the higher rate of DW application. On the contrary, the FA content was much lower in the residual year at both rates of DW. This may be attributable to its greater degradability and/or to its transformation to more complex molecules such as HAs (De Nobili and Petrussi., 1988; Fernández et al., 2007). Consequently, in the residual year the polymerization grade (PG) of DW-amended soils also increased significantly. These results suggest that repeated raw DW amendment can lead to an increase in the native soil organic matter stability by increasing the humified organic matter fraction, which represents a positive effect in the context of the beneficial recycling of DW. For that reason, it would be expected that DW amendment would be beneficial in the sense of improving soil properties and controlling degradation processes. This is of great importance because most agricultural soils in Mediterranean olive-oil producing countries are prone to progressive degradation (Antolín et al., 2005). Indeed, many authors argue that erosion is the principal problem associated with olive production (Fleskens and Stroosnijder, 2007), so that increasing organic matter and improving soil properties is more important than fertilizer application. Although in the residual year a sharp decrease in WSOC was observed in the DW treated soils, it is interesting to note that the DW54R treatment maintained WSOC values significantly above those of the control, and therefore DW could result in an increased risk of aquifer contamination, even two years after the last application.

Eight years of continued application of DW led to a significant linear increase of the total N in the soil (Table 2). Inorganic N concentrations (N–NO$_3$) also increased with increasing DW rates compared to the control. This result is in agreement with that reported following DW addition to the same soil in a short-term greenhouse and field experiment (López-Piñeiro et al., 2006, 2008). Similar trends were noticed in the residual year, confirming the observations of increased leaf N concentration following DW treatment, and showing that mineral N had not been immobilized during the degradation of the labile C constituents of the DW.

The available P content increased from low to very high levels (14.0–60.7 and 78.3 mg kg^{-1} for DW54C and DW54R, respectively), using very high and low in the sense of fertilizer recommendations (Table 2). The trend in available P was consistent with the increase in leaf P concentration following the DW application. The increase in soil available P content with DW application may not only provide agronomic benefits but also help resolve problems related to the P fixation frequently observed in calcareous soils (Sharpley et al., 1989). However, using the same soil in a short-term greenhouse experiment, López-Piñeiro et al. (2008) reported a significant decrease (from high to medium levels) in the available P due to DW addition. These results are indicative of the differences between controlled and field conditions when assessing the effects of organic amendment on soil properties, and confirms the need to conduct long-term studies to avoid drawing erroneous conclusions.

Compared to the control, increases of available K in the cumulative year were 3.0 and 5.7 times greater at the 27 and 54 Mg ha^{-1} DW rates, respectively (Table 2), reflecting the large amounts of K in the DW. These increases are consistent with the results of short-term studies using treated and untreated residues from three-phase (Madejón et al., 2003; Montemurro et al., 2004) and two-phase decanter processes: OW (Tejada and González, 2004; López-Piñeiro et al., 2006), and DW (López-Piñeiro et al., 2008). Increased available K was also detectable two years after the last DW application, confirming that this residue when directly applied to soil, even at the lower dose, could act as an alternative to

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K fertilizers. Moreover, the observed increase in available K could improve the tolerance of the olive trees to various stress situations, including drought (Tisdale et al., 1999) which is very frequent in the semi-arid Mediterranean areas.

Plots amended with the higher DW rate possessed pH values significantly lower than control plots, with decreases from 8.0 to 7.5 in both the cumulative and the residual years (Table 2). This reflects the strong buffering capacity of the soil under study (Tisdale et al., 1999). These results are consistent with those obtained in previous short-term studies (López-Piñeiro et al., 2008), where a significant ($P < 0.05$) relationship between the application rate of OW and pH was only found in acidic soils.

The application of raw DW also significantly increased the water soluble phenol (WSP) content (Table 2). Compared to the control, after eight years of repeated DW application, these increases were by factors of 2.8 and 6.3 for the DW27C and DW54C treatments, respectively. However, two years after the last DW application, a significant ($P < 0.05$) decrease of WSP concentration was observed in the amended soils. The average decreases for the amended plots were 43% and 55% for DW27C and DW54C, respectively, and only the plots amended at the higher rate (DW54R) had WSP values significantly higher than the control plots (T0R). The observed WSP decrease could be attributable mainly to microbial degradation and/or organic matter incorporation (Sierra et al., 2007). Nevertheless, and despite the high WSP concentration in the DW, the observed increases with both the cumulative and residual treatments were clearly insufficient to adversely affect crop growth. These results are consistent with the data reported by Mekki et al. (2007) who found that phenol compounds decreased rapidly in soils amended with untreated OW.

After eight years of repeated DW application to soil the electrical conductivity (EC) values were significantly greater in the amended than in the control plots (Fig. 1), as a consequence of the high EC values shown by DW (5.02 dS m^{-1}). A greater increase in EC values was observed in DW-amended soils in the residual than in the cumulative year. This fact could be attributable to the release of soluble organic and inorganic species during the humification of DW (Table 2), and could be related with the observed increase of nutrient availability in DW-amended soils, especially in the residual year. Indeed, EC was correlated positively and highly significantly ($P < 0.001$) with available P ($r = 0.908$) and K ($r = 0.934$), and significantly ($P < 0.05$) with NO_3–N. The observed EC increase suggests that yields of salt-sensitive crops might be affected negatively by DW application, which may therefore be a cause for concern, especially at the higher DW application rate.

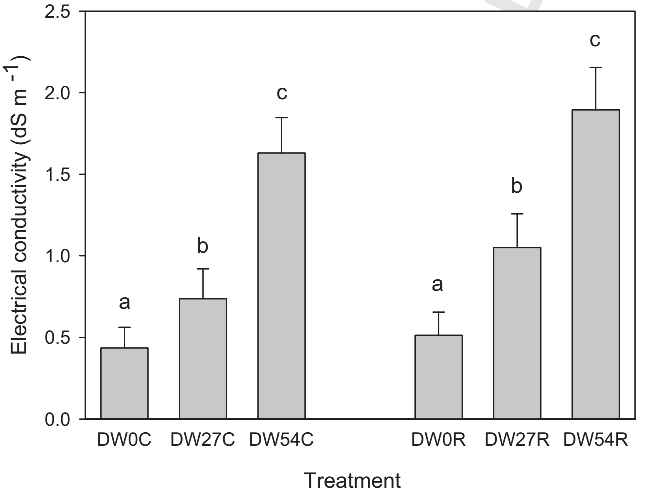

Fig. 1. Cumulative and residual effects of repeated applications of de-oiled two-phase olive mill waste on electrical conductivity. Bars with the same letter are not significantly different at $P < 0.05$ level of probability. Error bars represent one standard error of the mean.

3.3. Enzyme activities

None of the enzyme activities tested appeared to be negatively affected by the addition of the DW. On the contrary, these activities increased even at the higher DW application rates (Fig. 2). Dehydrogenase (DH) is considered to be a measure of a soil's microbiological activity (Moreno et al., 2009; Nannipieri et al., 2003), and can thus give information about the potential toxicity of DW (Benitez et al., 2004). A significant increase in DH activity occurred in the DW-amended soils in both the cumulative and the residual years (Fig. 2A). Compared to the control, DH activity increased by about 42% and 69% in the cumulative year and by about 19% and 44% in the residual year, at the 27 and 54 Mg ha^{-1} DW rates, respectively. This effect can be attributed to greater microbial biomass due to the addition of available organic substrates that can promote the growth of indigenous micro-organisms (Benitez et al., 2000). Similar responses in enzyme activities have been observed upon addition of olive mill wastes by several workers, although in short-term laboratory studies. Benitez et al. (2004) found that although DH activity at first decreased after raw OW application, it was immediately recovered after a short period of exposure, probably due to the increased pool of stabilized organic matter present in the OW-amended soils. The observed increase in DH activity also appears to be compatible with findings of Sampedro et al. (2009) who reported that raw DW application was not toxic to soil microorganisms despite the significant content of phenols in the DW-amended soils, and suggesting that pre-treatments of the DW, aimed at removing potentially toxic compounds, might not be necessary if the application rates were less than 54 Mg ha^{-1}.

Beta-glucosidase (GLU) plays an important role in hydrolytic processes during organic matter decomposition (Acosta-Martínez et al., 2008; Stott et al., 2010), and also provides information about the potential toxicity of the DW (Benitez et al., 2004). Compared to the control, in the cumulative year GLU activity was significantly ($P < 0.05$) higher in the DW-amended soils, irrespective of dose (Fig. 2B). However, in the residual year GLU activity increased with increasing DW application rate. According to Piotrowska et al. (2006), the increase of GLU in the DW-amended soils could indicate that the soil has gained a capability to utilize the carbohydrate material added with the DW. Furthermore, as GLU is mainly produced by fungi (Perucci, 1992), its increased activity suggests that the presence of DW caused a shift in the relative proportions of fungi and bacteria, especially at the higher DW application rate in the residual year (Fig. 2B). These results are in agreement with those obtained in short-term field (Mechri et al., 2007) and laboratory (Sampedro et al., 2009) studies in which a significant increase in fungi and its diversity was found after OW water and DW amendment, respectively.

De-oiled olive mill waste application significantly increased urease (UR) activity compared with the control (Fig. 2C), although the residual effects were much less pronounced than the cumulative effects. Thus, while in the cumulative year the increase of UR activity were 4.3 and 7.6 times greater at 27 and 54 Mg ha^{-1}, respectively, in the residual year these increases were only 1.1 and 2.1 times greater. The high concentration of available substrate coupled with the demand for nutrients by vegetation or micro-organisms could lead to a high activity of these enzymes during DW mineralization (Fernández et al., 2009; García-Gil et al., 2000). The inhibition of the UR activity observed in the amended soils in the residual year may be attributable to a higher concentration of metabolites such as NH_4^+ (Konig et al., 1966) released as a

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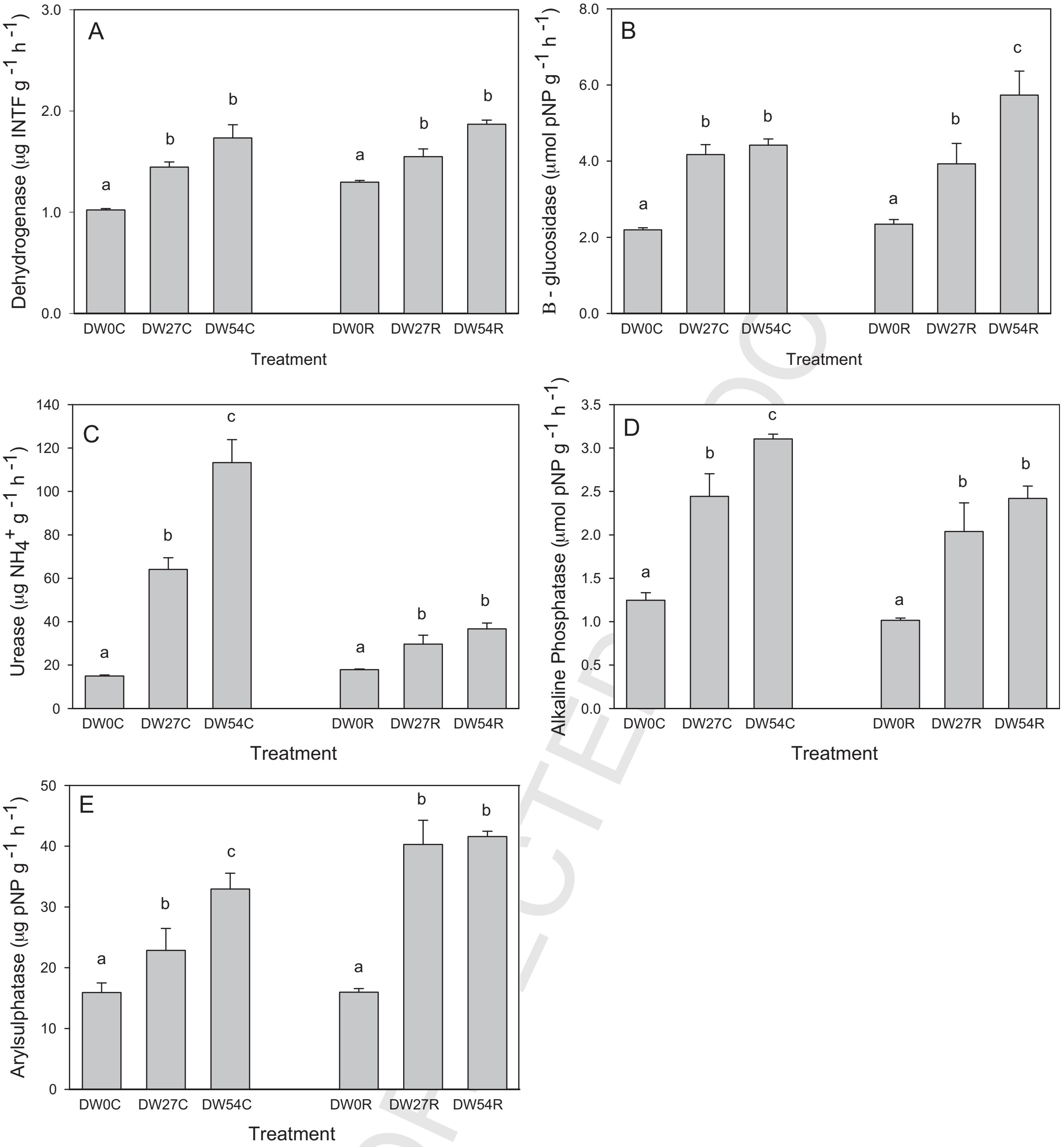

Fig. 2. Cumulative and residual effects of repeated applications of de-oiled two-phase olive mill waste on dehydrogenase (A), β-glucosidase (B), urease (C), alkaline phosphatase (D), and arylsulfatase (E). Bars with the same letter are not significantly different at $P < 0.05$ level of probability. Error bars represent one standard error of the mean.

consequence of the humification of DW, which was greater two years after the last waste addition (Table 2).

Soil phosphatase (PHO) activity, which plays an essential role in the mineralization of organic P, significantly ($P < 0.05$) increased after DW application (Fig. 2D). The increase in PHO activity can be explained by the fact that DW stimulates bacterial growth and enzyme production, including those involved in P turnover (Criquet and Braud, 2008; García et al., 1993). The PHO increases were similar to those reported using OW (Benitez et al., 2004) and DW (Sampedro et al., 2009), although these were short-term studies. However, in the residual year, one observes a tendency for decreasing PHO activity in the DW-amended soils compared to those in the cumulative year, especially at the higher DW application rate (Fig. 2D) for which the higher available P content was detected (Table 2). This is consistent with previous reports indicating that PHO can be decreased by an increase in available P, which could produce a feedback inhibition of this enzyme (Criquet and Braud, 2008; García-Gil et al., 2000).

A significant increase in arylsulfatase (ARS) activity was observed in the DW amended soils, although residual effects were more pronounced than the cumulative effects (Fig. 2E). Compared with the control, ARS activity increased by about 44% and 107% in the cumulative year, however in the residual year these increases were about 153% and 161% at the 27 and 54 Mg ha^{-1} DW application rates, respectively. Arylsulfatase activity was positively correlated with HA ($r = 0.907$, $P < 0.001$), PG (0.781, $P < 0.001$), and TOC ($r = 0.756$, $P < 0.01$). Significant correlations between enzyme activities and humic substances have been reported previously (Cayuela et al., 2008; Nannipieri et al., 1996). According to those authors, the protection of enzymes by humic-like substances could explain the high levels of ARS activity shown by DW-amended soils in the residual year.

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Positive relationships between soil enzyme activities and crop yields are to be expected (e.g., Fernández et al., 2009; Verstraete and Voest., 1977). In our study however, olive yields were only significantly correlated with UR ($r = 0.579$, $P < 0.05$), and PHO ($r = 0.520$, $P < 0.05$). This suggests that, although enzymes such as DH are considered to be good indicators of soil microbial activities, only some enzyme activities should be considered indicative of improved soil conditions for crop growth in DW-amended soils.

4. Conclusions

Long-term application of raw DW to an olive grove soil had positive effects on its chemical and biochemical properties without negatively affecting olive yields. The effects depended on the amendment rate, and especially on the degree of organic matter maturity. In particular, DW led to an increase of total organic carbon and humic fractions, which may contribute to improved soil workability and productivity. Moreover, the significant increase in dehydrogenase activity detected in the DW-amended soils suggests that this waste may not be toxic to soil microorganisms, at least at the application rates examined. Therefore, use of DW as an organic amendment in olive groves may be considered a viable option for the safe disposal of this waste and for the restoration of frequently degraded olive grove soils. This practice will also help to reduce the traditional use of this waste as fuel, thereby potentially lowering atmospheric CO_2 emissions and enhancing soil carbon sequestration. Nevertheless, the results suggest that DW application should not exceed 27 Mg ha^{-1} in order to mitigate the development of soil salinity. The long-term application of DW could also affect the soil's physical properties such as the appearance of hydrophobicity. There is thus an urgent need to conduct further research to determine the potential impact of DW on soil hydraulic properties (e.g., hydrophobicity, hydraulic conductivity, and infiltration rate). Such information would be useful to ensure that the use of DW as organic amendment is sustainable.

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